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Title: **METHOD FOR TRANSFORMING PLANT, THE RESULTANT PLANT AND GENE THEREOF**

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METHOD FOR TRANSFORMING PLANT, THE RESULTANT PLANT AND GENE THEREOF

Technical Field

This invention relates to a method for transforming a useful plant by introducing a gene of another species into the useful plant. More particularly, the present invention pertains the method for transforming the useful plant characterized in that the region of a factor relating to the poly (A) addition of the mRNA of the useful plant to be transformed contained in the base sequence of the gene of the other species is modified into another base sequence not relating to the poly (A) addition of the mRNA without substantially altering the function of the protein encoded by the gene to be introduced, the useful plant produced by it, a nucleic acid in which base sequence used thereto is modified, and a method for producing the said nucleic acid.

Background Arts

Growth of plants needs great numbers of nutrients. The plants absorb most of these nutrients necessary for growth from roots. The plants, which can not absorb nutrients in soil due to having hereditary low enzyme activities required for absorption of nutrients, are known.

For example, iron is an essential element for almost organisms, and is essentially required for large numbers of enzymes involved in functioning cells such as photosynthesis and respiration. Iron solubilized in soil exists mainly in the form of Fe(III) chelate [in some case, Fe(II) chelate]. In general, Fe(II) is prevalently absorbed as compared with Fe(III) by plants, but the absorption depends on plant species.

Plants have two types of mechanisms of iron uptake, i.e. absorption

mechanism (I) (refer to Fig. 1) and absorption mechanism (II) (refer to Fig. 2) (Mori, 1994).

The absorption mechanism (I) shown in Fig. 1 consists of: (1) release of proton into the rhizosphere (Olsen and Brown, 1980), (2) increased reducing activity of Fe(III) in cell membrane of roots (Brown et al., 1961 and Chaney et al., 1972), and (3) excretion of reduced and chelating substances from roots (Hether et al., 1981). Namely, Fe(III) is chelated by the released chelating substance, and Fe(III)-chelate in the free space of roots is reduced to Fe(II) on the cell membrane by ferric-chelate reductase and is absorbed through Fe(II) transporter. It is also thought that the proton is released into the rhizosphere and activity of reductase is increased by lowering pH in the free space. However, the problem is known that since reducing activity of Fe(III) is inhibited by higher pH, strong pH buffering action due to high concentration of carbonate anion results to cause lime chlorosis (Marchner et al., 1986).

Absorption mechanism (II) shown in Fig. 2 is specific to grass and is consisting of: (1) synthesis of mugineic acids (phytosiderophore), (2) release of mugineic acids into the rhizosphere, (3) formation of soluble complex of iron and mugineic acids, and (4) absorption of mugineic acids-iron complex by plants body (Takagi, 1976 and Takagi et al., 1984). The iron uptake mechanism by such the absorption mechanism (II) observed in grass has advantage not to be inhibited by higher pH.

Yeast (Saccharomyces cerevisiae), a model organism of eukaryote, performs iron absorption similar to the above absorption mechanism (I). Since in studies on the gene level in the higher plants, Fe(II) transporter has cloned by complementation of iron absorption mutant of yeast (Eida et al., 1996), no detailed mechanism of iron absorption has been studied.

Contrary to that, the mechanism in yeast (Saccharomyces cerevisiae) has

been studied in detail. Absorption of iron in yeast is initiated by a reduction of Fe(III) to Fe(II) by ferric-chelate reductase FRE1 and FRE2 (Dancis et al., 1990,1992, Georgatsou and Alexandraki, 1994). In the mechanism for uptake of reduced Fe(II) into cells, high affinity absorption mechanism and low affinity absorption mechanism are known.

In the absorption of iron by the high affinity absorption mechanism, after reoxidation of Fe(II) by multicopper oxidase FET3 (Askwith et al., 1994), Fe(III) may be incorporated into cells by ferric transporter FTR1 (Stearman et al., 1996). Copper is required in the reoxidation of divalent iron (Dancis et al., 1994, Klomp et al., 1997), and copper supplying pathway to FET3 has also studied (Yuan et al., 1995 and Lin et al., 1997).

Absorption of iron by the low affinity absorption mechanism may be performed by an action of Fe(II) transporter FET4 (Dix et al., 1994, 1997).

Such the iron absorption mechanism in yeast may be applied to plants, and plants which can be grown in the iron deficient soil may be created.

For that purpose, we have created transgenic tobacco, to which *FRE1* gene of yeast provided by Dr. Dancis (NIH) was transformed (Yamaguchi, 1995).

However, in the transgenic tobacco, to which *FRE1* gene was transformed, the reducing activity was not changed as compared with that of wild type. As a result of Northern hybridization analysis, the transcriptional product of yeast gene *FRE1* in tobacco was so small as 0.9 kb.

Example of such incomplete transcription, in which gene of another species is transformed into the higher plant, is gene group Cry encoding δ -endotoxin (insecticidal protein) of Bacillus thuringiensis. More than 42 Cry genes have been known and are classified into 4 classes (cryI - cryIV)(Whiteley and Schnepf, 1986). The

gene encoding this insecticidal protein was introduced into the higher plant, but neither expression nor extremely low expression was found.

This may be caused by (1) difference in codon usage, (2) high AT content in Cry gene, (3) unstable in mRNA, and (4) a partial splicing of Cry gene as intron.

A preparation of the transgenic plant with high expression of protein has been reported. Namely, in order to express Cry gene group efficiently in the higher plant, base sequence of Cry gene is modified to arrange with base sequence of the plant, and the primer is synthesized, then is completely synthesized by PCR (Perlak et al., 1991, Fujimoto et al., 1993, and Nayak et al., 1997).

Although transformation of the higher plant by introducing gene of the another organism species has known, the expression thereof was not sufficient. Various reasons have been provided as described in the above.

We have made extensive studies on factors for achieving the sufficient expression of a gene in a higher plant which has been transformed by introducing the above gene encoding a protein having a function carried by another organism so as to impart the function in the useful higher plant, and found that base sequence of the factor relating to the poly(A) addition of the mRNA of the transformed plant is an important part of the expression.

Consequently, the present invention provides a method for expressing the introduced gene in the transgenic higher plant with high efficiency, the said transgenic higher plant, and a method for modifying gene therefor.

Disclosure of the Invention

The present invention relates to a method for transforming a useful plant by introducing another gene into the useful plant characterized in that the region of a

factor relating to the poly(A) addition of the mRNA of the useful plant to be transformed contained in the base sequence of the said another gene is modified into another base sequence not relating to the poly(A) addition of the mRNA without substantially altering the function of the protein encoded by the gene to be introduced. Example of the region of a factor relating to the poly(A) addition of the mRNA is preferably AATAAA like base sequence, further the said region of a factor relating to the poly(A) addition of the mRNA is preferably the region existing downstream of the GT-rich base sequence. Further, modification of the base sequence of the said region is preferably carried out based on the codon usage of the transformed useful plant.

In the method of the present invention, it is preferable that base G and T rich region in the gene to be introduced is small; difference in content of base G and C within whole region of the gene to be introduced is small; the sequence has no ATTTA sequence; and/or upstream of the initiation codon of the gene to be transferred has Kozak sequence.

Further, the present invention relates to the transformed useful plant, which can be produced by the method of the present invention. The transformed useful plant of the present invention can be the organism and the seed and has no limitation in the form.

Further, the present invention relates to a nucleic acid, especially DNA, having the modified base sequence, which can be used by the above transforming method.

The base sequence of the nucleic acid of the present invention is a modified base sequence which can be expressed in the transformed useful plant with high efficiency, and, for example, is a factor relating to the poly(A) addition of mRNA of the said useful plant, and is characterized in that a part of factor relating to the said

poly(A) addition is replaced by the other base sequence, further the said base sequence has small G- and T-rich region of the base in the gene to be introduced, has small difference between G- and C-content of the base throughout the gene to be introduced, has no ATTTA sequence and/or preferably the upstream of the initiation codon of the gene to be introduced has Kozak sequence.

Further, the present invention relates to a method for production of the above nucleic acids characterized in that the above nucleic acids are divided into several fragments and these fragments are ligated.

Brief Description of Drawings

Fig. 1: Absorption mechanism (I) of iron in plants.

Fig. 2: Absorption mechanism (II) of iron in plants.

~~Fig. 3: A position of poly(A) addition in higher plant.~~

~~Fig. 4: G- and T-rich sequence in yeast gene *FRE1*.~~

Fig. 5: Schematic illustration of *refre1* synthesis.

~~Fig. 6: Sequence of 30 primers used in the synthesis of *refre1*.~~

~~Fig. 7: Relationship between *refre1* sequence and primer.~~

Fig. 8: Preparative scheme of full length *refre1*.

~~Fig. 9: Total sequence of designed *refre1*.~~

Fig. 10: Graphical illustration of G- and T-contents in *FRE1* (upper level) and *refre1* calculated by continued 8 base unit.

Fig. 11: Structure of binary vector pRF1.

Fig. 12: A photograph showing growth of transgenic plant of the present invention.

Fig. 13: A photograph showing anthesis of transgenic plant of the present

invention.

Fig. 14: Result of Southern hybridization of the transformant using *refre1* as a probe.

Left: Digestion by *Eco*RI and *Hin*dIII

Right: Digestion by *Hin*dIII

No.1 - No.2: Transformant

W.T: wild type

Fig. 15: Result of Northern hybridization of the transformant using *refre1* as a probe.

No.1 - No.2: Transformant

W.T: wild type.

Fig. 16: A photograph showing activity of ferric-chelate reductase in roots indicating red coloring of BPDS-Fe(II) complex by Fe(II).

Left: Wild type showing no red coloring.

Right: Transformant showing red coloring in roots.

Fig. 17: Photograph showing replicate experiment of the same as in Fig. 16 using another transformant. Red coloring is observed in the transformant (right).

Fig. 18: Photograph showing activity of ferric-chelate reductase by red coloring of BPDS-Fe(II) complex in roots, using second generation of plant obtained from seeds of the transformant. Red coloring of BPDS-Fe(II) complex is observed in the second generation of the transformant (left).

Best Mode for Carrying Out the Invention

The useful plants transformed in the present invention are no limitation, if these are industrially used plants such as foods and pharmaceuticals, and are

preferably higher plants such as grains, vegetables, fruits and tobacco.

Another gene introduced in the present invention is not limited, if it is useful for plants and has no detrimental effects for plants and human. It may be directly useful gene for plants and gene providing resistance against chemicals such as herbicide, and is preferably enzyme derived from organisms such as bacteria and yeast. For example, ferric-chelate reductase FRE1 of yeast involving absorption of iron is preferable.

We have found that in a transformed plant, factors affecting expression of introduced gene may be a base sequence which determines addition of poly(A) of mRNA. Further, we have found that in the upstream of the base sequence, which defines addition of poly(A), GT-rich base sequence is necessary. Namely, in the presence of GT-rich base sequence, addition of poly(A) is determined in plants, subsequently mRNA is split at the position after 10-30 bp from the poly(A) signal, for example AATAAA like base sequence, then poly(A) is added by an action of poly(A) polymerase. Accordingly, in case that the introduced gene has such the base sequence, in the transgenic plants, full length mRNA can not be expressed, and mRNA is split in the position after 10-30 bp from the poly(A) signal having AATAAA like base sequence.

Consequently, the present invention is characterized in that the poly(A) signal of plant in the introduced gene, for example AATAAA like base sequence, preferably GT-rich base sequence, is modified to another base sequence.

A method of design for modifying base sequence is, at first, codon is selected for not to change amino acid sequence encoded by gene to be introduced. Amino acid sequence can be changed, if the sequence has not substantial effect for function for protein, preferably the amino acid sequence may not be changed.

In case that multiple numbers of codons, which encode an amino acid, is

known, the codon having high rate of usage in the plant is preferably selected by considering the codon usage of the plant to be transformed.

Further, not only modification of base sequence of poly(A) signal but also deletion of GT-rich base sequence is preferable. Especially, in case that GT-rich base sequence exists with high proportion, since possibility of splitting mRNA in the region of poly(A) signal like base sequence appearing in the downstream of the GT-rich base sequence is high, a modification for reducing amount of GT content in such the region is important.

Further, in the present invention, in addition to the above modification, it is preferable to modify in order to make smaller difference between G- and C-content of bases throughout the full region of gene to be introduced. More preferably, the sequence should not contain ATTTA sequence, which is known as unstable sequence of mRNA, and/or the sequence has Kozak sequence, which is known as a sequence for effective translation of mRNA in the eukaryote, in the upstream of the initiation codon of gene to be introduced.

The method of the present invention includes a modification of base sequence combined further with usual method of modification to the above modification of base sequence.

The method for modification of base sequence can be made without limitation by known various methods. For example, any conventional method of modification by point mutation and splitting with restriction enzyme can be applied.

Further, in case that large numbers of base have to be modified or gene itself to be introduced has short length, it can be prepared by synthesis. As explained later concretely, even if length of gene is long, the gene is divided into several fragments, and each fragment, which is amplified by PCR, is ligated using restriction enzymes,

then gene having modified base sequence can be prepared.

The method of the present invention is further explained more concretely, but the method of the present invention can not be limited within the scope of the following explanation, and the broad application thereof based on the said explanation can be performed by the person skilled in the art.

We have tried to study the reason why length of mRNA of yeast *FRE1*, which was introduced into tobacco, was short (0.9 kb). As for the reasons for incomplete length of transcriptional product of yeast ferric-chelate reductase *FRE1*, which was introduced in tobacco, two possibilities were considered, i.e.

(1) a part of mRNA was spliced as intron, and

(2) a transcription was terminated within coding region.

As a result of further analysis by RT-PCR, it was found that poly(A) addition occurred within coding region in the transgenic tobacco, to which *FRE1* gene was transformed.

Example of the confirmed expression of yeast gene introduced into the higher plant is invertase (Hincha, 1996). In the present experiment, new knowledge, in which these is a case that full length mRNA can not be synthesized even in the same eukaryotic gene by introducing *FRE1* gene, could be obtained.

Reason why full length mRNA could not be synthesized in the *FRE1* transformed transgenic tobacco was addition of poly(A) within the coding region of *FRE1*.

A poly(A) site is not limited within one position, and in the upstream of each poly(A) site, AAUAAA like base sequence, putative poly(A) signal region was observed. However, although several AAUAAA like sequences were observed at 5'-site of *FRE1*, the poly(A) addition was not observed in these positions.

It may be a GU-rich sequence located in the upstream of the poly(A) signal to determine addition of poly(A) in plant. Namely, if GU-rich sequence exists, addition of poly(A) may be occured in the plant, and in the position of "PyA", which is located at the distance of 10-30 bp from the subsequently appeared AAUAAA like sequence, mRNA is splitted, then the poly(A) may be added by an action of poly(A) polymerase.

In conclusion, the fact that GU-rich sequence, which has no relation to addition of poly(A) in yeast, determines addition of poly(A) in plant, is a cause for not forming full length mRNA in the transgenic tobacco, to which FRE1 is transformed.

A sequence of ferric-chelate reductase *FRE1* having GT-rich region is shown in Fig.4. In Fig. 4, the boxed sequences are thought to be GT-rich regions.

As a result, in order to express ferric-chelate reductase FRE1 in tobacco, deletion of GT-rich sequence from *FRE1* gene may be effective. However, at present, as for the sequence, which determines addition of poly(A) in plant, there may be only known that a consensus sequence may be GU-rich and the sequence is not completely determined. So long as the exact consensus sequence has not be known, there may be possibility not to be obtainable the full length mRNA by only changing the sequence. We have, therefore, tried to design base sequence corresponding to codon usage of plants to be transformed without changing amino acid sequence of FRE1 in order to synthesize full length mRNA in plant.

In order to express yeast ferric-chelate reductase in tobacco, we have redesigned base sequence corresponding well to the codon usage of tobacco without changing amino acid sequence of FRE1. In design of base sequence, the following points are considered.

(1) GT-rich region is eliminated;

(2) Base sequence AATAAA, which may be a poly(A) signal, and the similar base

sequence are eliminated;

(3) In order to confirm easily the base sequence, restriction sites are set at the position in about every 400 bp (417-436 bp), and the sequence is divided in 5 segments;

(4) Base sequence, ATTTA sequence (Ohme-Takagi, 1993), which is called as unstable sequence of mRNA, is eliminated;

(5) In order not to make difference between base content G and C in whole region, position of codons are replaced; and

(6) Kozak sequence, which is a sequence for effectively translating mRNA in eukaryote (Kozak, 1989) is attached prior to the initiation codon.

The thus designed modified base sequence of yeast ferric-chelate reductase FRE1 is shown in Sequence listing, SEQ ID NO: 1. Amino acid sequence thereof is shown in SEQ ID NO: 2.

The designed gene is designated as reconstructed *FRE1* (hereinafter designates as "*refre1*").

The *refre1* of the present invention is synthesized by dividing into 5 segments (A-E) as shown in Fig. 5.

A segment A consists of a sequence of 1-434 bp, in which restriction sites are designed as in base 1: *Eco*RI, base 7: *Xba*I and base 429: *Bam*HI.

A segment B consists of a sequence of 429-845 bp, in which restriction sites are designed as in base 429: *Bam*HI and base 840: *Mro*I.

A segment C consists of a segment of 840-1275 bp, in which restriction sites are designed as in base 840: *Mro*I and base 1270: *Sal*I.

A segment D consists of a segment of 1270-1696 bp, in which restriction sites are designed as in base 1270: *Sal*I and base 1691: *Pst*I.

A segment E consists of a segment of 1691-2092 bp, in which restriction sites

are designed as in base 1691: *Pst*I, base 2081: *Sac*I and base 2087: *Hin*dIII.

Each segment A-E, each consisting of sequence having 417-436 bp, is synthesized using 6 primers having 77-83 mer, respectively. Thirty primers used, from A-1 to E-6, are shown in Fig. 6. These base sequence are shown in sequence listings, SEQ ID NO: 5 - SEQ ID NO: 34.

Among primers in the segments, -1, -2 and -3 are sense strands, and primers -4, -5 and -6 are anti strands. Primers are designed so as to have complementary base sequence consisting of 12 or 13 bp in the 3' end for primers -3 and -4, and overlapping sequence consisting of 12 or 13 bp in 3' end for primers -1 and -2, -2 and -3, -4 and -5, and -5 and -6. The primer -1 and -6 is designed to have restriction site at the base 1 in 5' end.

Relationship between these primers and the designed base sequences is shown in Fig. 7.

Respective segments A-E are prepared by PCR using primers synthesized according to the above base sequence (refer to Fig. 5).

After the reaction mixture of third step PCR was electrophoresed with 0.8% agarose gel, bands having expected length (417 - 436 bp) were cut and purified, then were cloned into plasmid pT7Blue (R) vector (supplied by Takara Corp.). The base sequences of the obtained clones were confirmed and the clones having exact base sequences were selected by applying fluorescent DNA sequencer DSQ-1000L (made by Shimadzu Corp.).

Segments having exact sequences were obtained and full length *refre1* was prepared by applying restriction sites according to methods shown in Fig. 8.

Direction of insertion in segments B and E is essentially required for preparation of full length. In other segments, the segments containing exact base

sequence were used without relation to direction of the insertion.

Total base sequence of the obtained *refre1* is shown in Fig. 9. Specific features of sequence of *refre1* are:

(1) 75.3% of homology to the original *FRE1* (100% homology in amino acid sequence);

(2) To have no sequence consisting of only G or T which is continuously linked more than 8 bases;

(3) It does not contain not only a sequence AATAAA but also sequences replaced by any one of bases in the above sequence;

(4) It does not contain a sequence ATTTA; and

(5) No difference is observed in GC content through the whole region of the sequence.

Fig. 10 shows decreased numbers of sequences consisting of serial G and T in *refre1* as compared with the original *FRE1*. This illustrates GT content of serial 8 bases in the *FRE1* and *refre1* sequences. As shown in Fig. 10, uniformity of GT content in *refre1* is demonstrated as compared with the original *FRE1*.

The thus synthesized gene *refre1* is introduced into tobacco (Nicotiana tabacum L. var. SRI). As a result of transformation, 68 kanamycin resistant plants were reproduced. In order to confirm transformation of the objective gene in the reproduced plant and its copying number, genomic Southern hybridization was conducted. As a result, one to several copied plants of the transformant, *refre1* gene was confirmed.

A method from gene introduce into plant cells to reproduction of plant can be performed by conventional method, for example, as described in "Laboratory Manual on functional analysis of plant gene" (Maruzen) [ref. (4)].

Specifically, a fragment of restriction enzymes, *Xba*I and *Sac*I, in *refre1*, which was cloned with pT7Blue(R) vector by the above method, was exchanged with ORF of

β-glucronidase in the binary vector pBI121 (TOYOBO Co. Ltd.) to prepare binary vector pRF1. The structure of the binary vector pRF1 is shown in Fig. 11.

The thus obtained binary vector pRF1 was transformed into E. coli, and E. coli, which is bearing helper plasmid pRK2013, were shake cultured at 37°C for overnight. On the other hand, Agrobacterium tumefaciens C58 was shake cultured in LB liquid culture medium 1 ml containing proper antibiotic at 26°C for 2 nights. Each 100μl thereof was mixed on LB plate without containing antibiotic, cultured at 26°C for 2 nights, then surface of the plate was scraped by using platinum spatula, and cultured on the selection plate [LB plate containing 100μg/μl rifampicin (Rf) and 25μg/μl kanamycin (Km)] at 26°C for 2 nights to form single colony.

The single colony was shake cultured in LB (Km and Rf) liquid medium 4 ml at 26°C for 2 nights. Plasmid was extracted, and restriction enzyme treated cleavage pattern of the plasmid indicated existence of pRF1.

Plant to be transformed was prepared as follows.

Two or three young leaves, size about 8 cm, of wild type tobacco were cut, and were sterilized in a petri dish, filled with sterilized solution (hypochlorous acid 10% and Tween 20, 0.1%), with stirring for 15 minutes. After rinsing three times with sterilized water, the leaves were cut off in 8 mm squares. To the leaves in a petri dish was added the cultured liquid 3 ml of the binary vector bearing Agrobacterium tumefaciens C58, which was cultured 26°C for 2 nights. After 1 minute, the liquid was rapidly removed by using Pasteur pipette, and residual liquid was removed off on the autoclaved filter paper.

Fragments of leaves were put on a culture medium, added with benzyl adenine and naphthaleneacetic acid to the MS medium, and cultured under light

condition at 25℃ for 3 days. Thereafter, the fragments of leaves were transferred to the medium added with CLAFORAN, and cultured for 1 week further were transferred to the medium added with CLAFORAN and kanamycin, then inoculated in every 2 weeks. When calli were induced and shoots were formed, the shoots were cut off with scalpel and were transferred to the MS medium added with kanamycin.

Shoots with roots were transplanted to the vermiculite and the plants were raised with supplying hyponex (Hyponex Japan Co. Ltd.) to obtain the transgenic plants.

Sixty-eight transgenic plants having kanamycin resistance could be obtained as a result of transformation. Example of photograph of the grown plant is shown in Fig. 12 and the photograph of plant with flower is shown in Fig. 13.

Among them, 5 individual plants were treated with genomic Southern hybridization. Result is shown in Fig. 14.

In the genomic Southern hybridization, extraction of genomic DNA from the transgenic tobacco was performed according to the description in "Plant Cell Technology Series 2, Protocol for PCR Experiments of Plants" (Shujun-Sha) [Ref. (2)]. The obtained genomic DNA was digested by restriction enzymes *Eco*RI and *Hin*dIII and the hybridization was performed by using a probe, which was prepared with full length fragment of *refre1* as a template ([α-32P]-dATP was used).

In the genomic Southern hybridization shown in Fig. 14, amounts of DNA were arranged at the time of restriction enzyme treatment, but deviation was observed due to treating with ethanol after restriction enzyme treatment. Consequently, darkness of bands detected is not always reflecting the copy numbers of the introduced gene.

In digestion with restriction enzymes, *Eco*RI and *Hin*dIII, band, size 3.2 kb,

which was expected in all individuals, was observed. However, in the individual No. 12, a band with slightly smaller than 3.2 kb was detected. According to this result, 1 copy of *refre1* in No. 1 and No. 11, 3 or 4 copies in No. 2 and 4 copies in No. 9 were thought to exist.

In the digestion by *Hin*dIII on No. 12, band could not be detected due to loading failure on the gel.

As a result of the above genomic Southern hybridization analysis, the *refre1* gene was found to be introduced into the selected five individuals.

When the sequence is cleaved by restriction enzymes *Eco*RI and *Hin*dIII, a sequence from promoter to terminator is cleaved, and the introduced *refre1* gene is transcribed to mRNA under regulation of CaMV35S promoter. In the *Eco*RI and *Hin*dIII digestion of No. 12, a reason for detecting a band slightly smaller than 3.2 kb might be due to the fact that one of the introduced construction was cleaved before integration in the plant genom, and was inserted into the position close to *Eco*RI or *Hin*dIII site in the plant genom.

Next, in the transformed tobacco No. 1 and No. 2, in which the introduced *refre1* gene was confirmed by the genomic Southern hybridization hereinbefore, formation of full length mRNA was confirmed by Northern analysis.

In the Northern analysis, a method of blotting was performed, for example, according to the conventional method described in "Cloning and Sequencing" (Noson-Bunka-Sha) [Ref. (1)], and the method in hybridization was performed according to the method as described in Southern analysis hereinbefore.

A result of Northern hybridization is shown in Fig. 15. In Fig. 15, no band is detected in the lane of wild type (W.T.). In lanes of No. 1 and No. 2, major bands with a size of 2.5 kb are detected and several bands smaller than that are detected.

In Northern analysis, formation of the full length mRNA as a result of introducing *refre1* of the present invention could be confirmed. In the present analysis, fundamentally although total RNA should be extracted from the root, in which mRNA is expected to be expressed, in this experiment, if the root is cut, then the plant of the subsequent generation can not be obtained, consequently the extraction has to perform from leaves. Since CaMV35S as a promoter is used and *refre1* gene is expressed in the whole parts of plant, even in the analysis performed by extraction of total RNA from leaves, it is confirmed that the transcription can be performed in leaves and roots of plants and site of addition of poly(A) is not changed.

As a result of Northern hybridization, since the transcriptional product of 2.5 kb was confirmed in the tobacco, to which *refre1* was introduced, poly(A) addition might occur by NOS terminator. Full length mRNA was also found in the tobacco, to which *refre1* was introduced.

A band smaller than the length of 2.5 kb as seen in Fig. 15 is detected at the position corresponding to rRNA as compared with that of photograph after electrophoresis. Although it was thought to be a nonspecific absorption of probe in rRNA, since it was not hybridized with wild type RNA, it is surely hybridized with transcriptional product of *refre1*. There may be possibly produced the shorter mRNA than the full length mRNA in the *refre1*. However, since it is detected as the same length with rRNA, it may be thought that, in the electrophoresis of RNA, mRNA of *refre1* may be dragged by rRNA, which exists in large amount. This reason may be clarified by Northern hybridization with purified poly(A) + RNA, however as obviously shown in Fig. 15, most of mRNA is full length mRNA and there is no reason to trace and clarify such the reason.

In order to perform Northern hybridization, RNA of the transformant of No. 1,

which was found as one copy, and that of the transformants of No. 2, which were found as 3 or 4 copies, were electrophoresed, it was found that the bands of No. 2 were dark colored depending on copy numbers of *refre1* gene.

Further, among the obtained transformed 68 plants of tobacco (selected by kanamycin), constant ferric-chelate reductase in root was confirmed in 6 plants.

For detection of reductase activity, a property of red color formation of the complex of bathophenanthroline disulfonic acid (BPDS), which is a strong chelater for Fe(II), with Fe(II) was applied. After removal of vermiculite from transformant and wild type tobacco, roots were laid on the gel containing BPDS with shield light using aluminum foil and stood at 27℃ for 24 hours. Reduction of Fe(III) was confirmed by coloring in the rhizosphere of the transformant.

Photographs confirming reductase activity are shown in Fig. 16 and Fig. 17. In photographs of Fig. 16 and Fig. 17, red coloring is observed in the transformant of the right photographs as shown with black color.

As shown, ferric-chelate reductase was detected in all of 6 plants (selected by kanamycin), which were used for confirmation of ferric-chelate reductase activity in roots. In order to demonstrate difference between the transformed tobacco and wild type tobacco, reaction time of reductase was set for long time as 24 hours, but the difference was observed at about 1 hour of the reaction time. In all of 6 plants used in the transformation experiments, the leaves, which were put on the gel for detecting activity, showed tendency of crinkle as compared with the condition of wild type leaves. This may be due to involvement in the mechanism of the introduced *refre1* gene expression in the leaves. Though not so many times of activity tests were performed because of this phenomenon, it is clear that *refre1* gene is expressed as a result of transcription and translation in the root under regulation of CaMV35S promoter.

As explained in the above, we have created novel tobacco which could express yeast ferric-chelate reductase FRE1 in the higher plant tobacco.

Reasons for not obtaining full length transcription product of different organism gene may be due to two possibilities including splicing a part of mRNA as an intron, and adding poly(A) within the coding region.

The present invention provides a method for designing base sequence for obtaining full length transcriptional product by transferring gene of different species in the higher plant. In the method of the present invention, in order to avoid addition of poly(A) in the coding region, it was found that it is necessary to design the sequence consisting of continued base sequence of 8 bases or more without containing sequence consisting of only G or T, and to design the sequence without containing not only a sequence of AATAAA but also a sequence, in which any one of bases thereof is replaced by another base (i.e. NATAAA, ANTAAA, AANAAA, AATNAA, AATANA, or AATAAN).

It was also found that to design the sequence, in which G and C contents should be constantly distributed in the full region, is important.

Further, in the concrete explanation of the present invention hereinbefore, since CaMV35S was used as a promoter, ferric-chelate reductase was expressed in the transformed tobacco of the whole plant. As shown, locally expressing gene can be expressed in the systemic plant as a result of combining with the promoter. On the contrary, the expressing gene in the systemic plant can be expressed in the local region by using combination with preferable promoter.

A mechanism of absorption by reduction of Fe(III) is specific to iron acquiring mechanism in the monocots and dicots except for grass, and also the grass may absorb iron by reducing Fe(III) to Fe(II) under the condition of sufficient iron. As a result of ligating the ferric-chelate reductase gene *refre1* of the present invention with a

promoter, which is specifically active in the root under iron deficient condition, novel grass, in which iron absorption mechanism (I) and absorption mechanism (II) under the condition of iron deficiency can be functioned, may able to be created.

References in the present invention are listed hereinbelow.

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Examples

The present invention will be explained in detail hereinbelow in examples, but is not construed as limiting within these examples.

In the examples hereinbelow, fundamental gene manipulation is performed

according to the description of "Cloning and Sequencing" (Noson-Bunka-Sha) and analysis of base sequence of gene is performed by using DNASIS (made by Hitachi Corp.).

Example 1 (Extraction of total RNA from *FRE1* introduced transgenic tobacco)

Extraction of total RNA from *FRE1* introduced transgenic tobacco was performed according to a method described in the reference (Naito et al., 1988).

Leaves 2g of *FRE1* introduced transgenic tobacco were put in the mortar, and liquid nitrogen was added thereto, then leaves were completely mashed. Three fold amounts of buffer for extraction and equal amount of phenol/chloroform (1 : 1) were added to the debris and suspended, then centrifuged at 8000 rpm for 15 minutes, and extracted with chloroform once. Ethanol precipitation was conducted at -80°C for 30 minutes, and centrifuged at 4°C for 30 minutes. Precipitate was washed with 70% ethanol and dried in vacuum. The precipitate was dissolved in DEPC treated water 1 ml, centrifuged at 13500 rpm for 3 minutes, and the supernatant was transferred to a new tube, further 10 M LiCl, 1/4 volume, was added and allowed to stand on ice for 2 hours. The mixture was centrifuged at 12000 rpm at 4°C for 10 minutes, then the precipitate was washed with 70% ethanol and dried in vacuo. The dried product was dissolved in DEPC treated water 50μl.

Reagent buffer for extraction

1M Tris HCl pH 9.0

1% SDS

(β-mercaptoethanol 120μl was added to 6 ml of buffer before use)

Example 2 (Purification of poly(A) + RNA and synthesis of cDNA)

Poly(A) + RNA was purified from total RNA 100μg obtained in example 1 by applying with Dynabeads Oligo (dT) 25 (DYNAL Inc.). This poly(A) + RNA was treated with reverse transcription reaction by M-MLV reverse transcriptase (TOYOBO Co. Ltd.) at 37℃ for 1 hour using the following hybrid primer to obtain cDNA.

Hybrid primer (dT^{17} adapter primer):

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'

Example 3 (RT-PCR and confirmation of base sequence)

PCR was conducted with the primer specific to hybrid primer and the 5' primer of *FRE1* using cDNA obtained in example 2 as a template.

Reaction product of PCR was electrophoresed with 0.8% agarose gel, and the obtained band was cloned to pT7Blue(R) vector (Takara Corp.). Colony was shake cultured in LB medium for overnight, extracted the plasmid by alkaline-SDS method, and the base sequence of 7 clones, to which the insertion was confirmed by restriction enzyme treatment, was determined by using Bca BEST DNA polymerase ("Biotechnology Experiments Illustrated, Fundamentals of gene analysis") (Shujun-Sha).

Primer specific to hybrid primer:

5'-GACTCGAGTCGACATCG-3'

5' primer of FRE1:

5'-ACACTTATTAGCACTTCATGTATT-3'

Reaction condition for PCR:

(1) 95℃ for 5 minutes;

(2) 95℃ for 40 seconds;

(3) 55℃ for 30 seconds;

(4) 72℃ for 1 minute;

(5) 72℃ for 10 minutes; and

(6) 4℃

In the above procedures, (2), (3) and (4) were repeated 40 times.

As a result, in the transgenic tobacco transformed with *FRE1*, poly(A) was attached at the position as shown in Fig. 3, in the transcribed mRNA from *FRE1* gene.

Attached points of poly(A) were not uniform, and several length of mRNA was observed. A sequence, which might be recognized as poly(A) signal at the upstream of poly(A) site, was indicated as putative poly(A) signal.

Example 4 (Production of each segment by PCR)

Each segment was prepared by PCR as illustrated in Fig. 5. The super Taq (Sawady Inc.) was used as Taq polymerase.

Composition of PCR reaction is as follows.

PCR reaction solution in the first step:

10 x buffer	10 μl
2 mM dNTP mixture	10 μl
20 μM primer (-3)	5 μl
20 μM primer (-4)	5 μl

distilled water to total volume 99.5 μl

PCR reaction solution in the second step:

PCR reaction solution in the first step	1 μl
10 x buffer	10 μl

2 mM dNTP mixture	10 μl
20μM primer (-2)	5 μl
20μM primer (-5)	5 μl
distilled water to total volume	99.5 μl

PCR reaction solution in the third step:

PCR reaction solution in the second step	1μl
10 x buffer	10μl
2 mM dNTP mixture	10 μl
20μM primer (-1)	5 μl
20μM primer (-6)	5 μl
distilled water to total volume	99.5 μl

Reaction conditions for PCR:

(1) 95℃ for 5 minutes;

(2) add Taq 0.5 μl

(3) 95℃ for 40 seconds;

(4) 45℃ for 1 minute;

(5) 72℃ for 1 minute;

(6) 94℃ for 40 seconds;

(7) 60℃ for 30 seconds;

(8) 72℃ for 1 minute;

(9) 72℃ for 10 minutes;

(10) 4℃

The above procedures of (3), (4) and (5) were repeated 5 times, and the procedures of (6), (7) and (8)) were repeated 20 times, respectively.

Example 5 (Cloning and confirmation of base sequence)

After electrophoresis of PCR reaction solution in the third step in example 4 with 0.8% agarose gel, a band, which had expected length (417 - 436 bp), was cleaved and purified, then was cloned into the plasmid pT7Blue (R) vector (Takara Inc.). The base sequence of the thus obtained clone was confirmed and the exact base sequence was selected using SHIMADZU luminescent DNA sequencer DSQ-1000L.

After obtaining segment of each exact sequence, full length of *refre1* was prepared as shown in Fig. 8 by applying with restriction enzyme sites. A direction of insertion of the segment B and E was essential for preparing the full length. As for the other segments, the sequence containing exact base sequence was used without relation to the direction of insertion.

Full length of base sequence of the synthesized *refre1* is shown in sequence listing SEQ ID NO: 1 and Fig. 9.

Example 6 (Introduction of *refre1* into tobacco)

A gene *refre1* synthesized in example 5 was introduced into tobacco (*Nicotiana tabacum* L. var. SRI). As a result of transformation, 68 individual plants resistant to kanamycin were generated. Genomic Southern hybridization was performed in order to confirm introduction of *refre1*, an objective gene, in the generated plant and copying number thereof. As a result, existence of one to several copies of *refre1* gene was confirmed.

A method from gene introduce into plant cells to generation of plant was performed according to description in "Laboratory Manual for Functional Analysis of Plant Genes" (Maruzen).

(1) Preparation of binary vector pRF1 for transformation

*Xba*I and *Sac*I fragments of *refre1*, which were cloned in pT7Blue (R) vector, were exchanged with ORF of β-glucronidase of binary vector pBI121 to prepare a binary vector pRF1. A structure of the binary vector pRF1 is shown in Fig. 11.

(2) Transfer of binary vector pRF1 into Agrobacterium

Agrobacterium tumefaciens C58 was shake cultured at 26℃ for 2 nights in LB liquid medium 1ml containing suitable antibiotic, and E. coli having pRF1 and E. coli having helper plasmid pRK2013 were shake cultured at 37℃ for one night in LB liquid medium 1ml containing suitable antibiotic. Each 100 μl was mixed on the LB plate without containing antibiotics. After the mixture was cultured at 26℃ for 2 nights, plate surface was scraped out the plate using platinum loop and incubated to form single colony on the selection plate [LB plate containing 100 μg/μl rifampicin (Rf) and 25 μg/μl kanamycin (Km)] (at 26℃ for 2 nights).

The thus obtained single colony was shake cultured in LB (Km and Rf) liquid medium 4 ml at 26℃ for 2 nights, and the plasmid was extracted by alkaline-SDS method, then existence of pRF1 was confirmed by observing cleavage patterns by restriction enzymes.

(3) Infection of Agrobacterium to tobacco and regeneration of plant

Two or three young leaves of tobacco (Nicotiana tabacum L. var. SRI), size about 8 cm, were cut, put them into the petri dish filled with sterilized water (hypochlorous acid 10% and Tween 20, 0.1%), and sterilized with stirring for 15 minutes. The leaves were rinsed with sterilized water for 3 times, and were cut in 8

mm square using scalpel. Cultured liquid of Agrobacterium 3 ml having binary vector pRF1 cultured at 26℃ for 2 nights was added to fragments of leaves in the petri dish.

After one minute, the liquid was immediately removed off by using Pasture pipette and the residual liquid was removed off using autoclaved sterilized filter paper. The leaves were put on the MS medium (II) hereinbelow and cultured at 25℃ for 3 days under lighting condition. Thereafter fragments of leaves were transferred to MS medium (III) and cultured for 1 week, then transferred to the MS medium (IV) and subcultured in every 2 weeks. When calli were induced and shoots were formed, the shoots were cut using scalpel and transferred to the MS medium (V). The shoots with roots were inoculated to vermiculite, and raised with supplying hyponex (Hyponex Japan Co., Ltd) to obtain the regenerated plant.

The compositions of MS medium for tobacco used in the experiments hereinbefore are as follows.

Major elements (g/l)

NH_4NO_3	1.65
KNO_3	1.9
$CaCl_2 \cdot 2H_2O$	0.44
$MgSO_4 \cdot 7H_2O$	0.44
KH_2PO_4	0.17

Minor elements (mg/l)

H_3BO_4	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83

$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Fe(III)Na-EDTA	0.042 mg/l
myo-inositol	100 mg/l
thiamine	5 mg/l
sucrose	30 g/l
geranylated g	2 g/l

MS medium (I) was prepared by the composition hereinbefore. The other MS media were prepared by adding the following phytohormone and/or antibiotics to the MS medium (I).

Phytohormone	benzyladenine (BA)	1.0 mg/l
	naphthaleneacetic acid (NAA)	0.1 mg/l
Antibiotics	kanamycin	100 mg/l
	claforan	200 mg/l

MS medium (II) MS medium (I) + BA + NAA

MS medium (III) MS medium (I) + BA + NAA + claforan

MS medium (IV) MS medium (I) + BA + NAA + claforan + kanamycin

MS medium (V) MS medium (I) + kanamycin

Example 7 (Southern analysis)

(1) Extraction of genomic DNA from tobacco

Extraction of genomic DNA from tobacco was performed according to the method described in "Plant Cell Engineering Series: Protocol for PCR Experiments in

Plants" (Shujun-Sha).

Leaves 0.1 - 0.2g were put in the mortar, and liquid nitrogen was added thereto, then leaves were completely mashed. The crushed leaves were put into the Eppendorf tube, and 2% CTAB solution 300 μl was added and mixed, then treated at 65℃ for 30 minutes. Equal amount of chloroform and isoamyl alcohol (24 : 1) was added and mixed for 5 minutes.

The mixture was centrifuged at 12000 rpm for 15 minutes, and the upper layer was transferred to the new tube, then chloroform-isoamyl alcohol extraction was repeated once again, and the upper layer was transferred to the new tube. 1 - 1.5 volume of 1% CTAB solution was added, mixed, allowed standing at room temperature for 1 hour, and centrifuged at 8000 rpm for 10 minutes. The upper layer was discarded and 1M CsCl 400 μl was added to the residue, and heated at 65℃ until complete dissolving the precipitate. 100% ethanol 800μl was added thereto, mixed, allowed to standing at -20℃ for 20 minutes, then centrifuged at 12000 rpm for 5 minutes. The upper layer was discarded, and the residue was washed with 70% ethanol, dried in vacuum and dissolved in TE buffer 30μl.

Reagents 2% CTAB solution

Tris-HCl (pH 8.0)	100 mM
EDTA (pH 8.0)	20 mM
NaCl	1.4 M
CTAB (cetyltrimethylammonium bromide)	2 %

1% CTAB solution

Tris-HCl (pH 8.0)	50 mM
EDTA (pH 8.0)	20 mM

CTAB	1 %

(2) Cleavage of genomic DNA by restriction enzyme and electrophoresis

Restriction enzyme treatments were performed by digestion using *Eco*RI and *Hin*dIII, by which sequence from pCaMV35S to tNOS was cleaved, and by digestion using only *Hin*dIII, by which sequence of upstream of pCaMV35S was cleaved.

Genomic DNA 10 μg with the reaction volume 100 μl was treated by restriction enzyme for overnight, precipitated by adding ethanol and dissolved the precipitate in TE buffer 20μl. To the solution was added the loading buffer 2μl, and the solution was electrophoresed with 0.8% agarose gel at 60V for 5 hours. After completion of electrophoresis, gel was stained with ethidium bromide and photographed on the UV transluminater with the scale.

(3) Blotting and hybridization

Gel after photographing was washed with distilled water, and was shaken in 0.2 N HCl for 10 minutes. A method of blotting was performed according to the description in "Cloning and Sequencing" (Noson-Bunka-Sha). The gel was transferred to nylon membrane (New Hybond-N+ Amersham) with 0.4 N NaOH, and the membrane washed with 2 x SSPE for 5 minutes, and dried at room temperature for 3 hours. A method of hybridization was referred with "Biotechnology Experiments Illustrated, Fundamentals of Gene Analysis" (Shujun-Sha) The membrane was treated for prehybridization with prehybridization buffer 30 ml, which was previously warmed at 65℃, for 1 hour at 65℃, and the hybridization buffer was exchanged (25 ml). Probe was added and hybridization was performed at 65℃ for 12 hours. The membrane was washed with washing solution, which was previously warmed at 65℃,

twice at 65℃ for 10 minutes, and was washed once with high stringent washing solution at 65℃ for 10 minutes. The membrane was wrapped with Saran wrap, exposed on imaging plate for 24 hours, and result was confirmed by image analyzer (Fuji Photo Film Co. Ltd.).

Reagents

20 x SSPE

NaCl	3 M
NaH_2PO_4	0.2 M
EDTA	1 mM

1M Church phosphate buffer

$NaHPO_4$ 0.5 mol was added to distilled water about 800 ml, adjusted pH to 7.2 by H_3PO_4, then filled up to 1 liter by adding distilled water, and autoclaved.

Hybridization buffer

Church phosphate buffer	0.5 M
EDTA	1 mM
SDS (v/v)	7 %

Denatured salmon sperm (1 mg/ml) 1/100 vol. was added before use.

Washing solution

Church phosphate buffer	40 mM
SDS (v/v)	1 %

High stringent washing solution

0.2 x SSPE	
SDS (v/v)	0.1 %

(4) Preparation of probe

Probe was prepared by random primer DNA labeling kit ver. 2.0 (Takara Corp.) using full length *refre1* as a template (proviso that [α-^{32}P]-dATP was used), and non-reacted [α-^{32}P]-dATP was removed using Probe Quant TM G-50 Micro Columns (Pharmacia, Biotech Inc.).

Result is shown in Fig. 14. The left side in Fig. 14 shows digestion using restriction enzymes *Eco*RI and *Hin*dIII, and the right side in Fig. 14 shows digestion using only *Hin*dIII. In Fig. 14, W.T. means wild type.

Example 8 (Northern analysis)

(1) Extraction of total RNA

Total RNA was extracted from leaves of the transgenic tobacco, to which *refre1* gene was introduced, and leaves of the wilt type tobacco according to the same method as described in example 1.

(2) Electrophoresis of RNA

Electrophoresis vessel, gel receiver, comb and Erlenmeyer flask were treated previously with abSolve (RNase inhibitor, Du Pont Inc.). 20 x MOPS 10 ml, agarose 2.4 g and sterilized distilled water 100 ml were poured into Erlenmeyer flask, and agarose was dissolved using microwave oven. Formaldehyde 10 ml was added to the gel which was cooled to about 50℃, and sterilized distilled water was added up to 200 ml, which was used when gelification occurred. 1 x MOPS about 800 ml was added in the electrophoresis vessel, and added ethidium bromide 10 mg/ml thereto for use as electrophoresis buffer. RNA sample buffer 16 μl was added to the total RNA 10 μg, filled up to 20 μl with sterilized distilled water, and the mixture was warmed at 65℃

for 10 minutes, then allowed to standing for 5 minutes on ice, and was electrophoresed. Electrophoresis condition was that after electrophoresis was performed at 60 V for 1 hour, further electrophoresis was performed at 120 V for 2 hours.

Reagents:

20 x MOPS

MOPS	0.4 M
NaOAc	0.1 M
EDTA	0.02 M

RNA sample buffer

Formaldehyde	1.6 ml
Formamide	5.0 ml
20 x MOPS	0.5 ml
glycerol pigment solution	1.6 ml
Total	8.7 ml

Glycerol pigment solution

glycerol	5 ml
bromophenol blue	1 mg
xylenecyanol	1 mg
0.5 M EDTA (pH 8.0)	0.02 ml

(3) Blotting and hybridization

After electrophoresis, gel was set on UV illuminator and photographed with the scale. A method of blotting was followed according to the description in "Cloning

and Sequencing" (Noson-Bunka-Sha). Namely, RNA was transferred from gel to nylon membrane (New Hybond-N, Amersham Inc.) with 20 x SSPE. After 12 hours, the membrane was washed with 2 x SSPE for 5 minutes, and dried at room temperature for 3 hours, then RNA was fixed on the membrane by irradiating with UV for 5 minutes.

A method of hybridization was performed as same as the case of Southern analysis.

Result is shown in Fig. 15. In Fig. 15, W.T. indicates wild type. No band was detected in the lane of wild type (W.T.). In No. 1 and No. 2 lanes, major band was detected at the size of 2.5 kb, and several bands were detected in the lower position thereof.

Example 9 (Confirmation of ferric-chelate reductase)

The transgenic tobacco, to which *refre1* gene was introduced, and wild type tobacco were transplanted in vermiculite and raised with supplying hyponex. Ferric-chelate reductase activity was confirmed by using plants, about 5 cm - 10 cm.

For confirmation of ferric-chelate reductase activity, red coloring generated by formation of complex with bathophenanthroline disulfonic acid (BPDS), which was strong chelating agent for Fe(II), and Fe(II) was applied. Agarose was added to assay buffer up to 0.4%, dissolved by using microwave oven, and cooled. 500 μM Fe(III)-EDTA, 1/100 vol., and 500 μM BPDS, 1/100 vol., were added to the slightly cooled gel, and stirred to put in the vessel, then waited for solidification. After removed off vermiculite from the transformant and wild type tobacco, roots were laid on the gel, and shielded from light and allowed to standing at 27℃ for 24 hours.

The similar experiment was performed using second generation of the

transgenic plant, which was germinated from seeds of the regenerated plant. Reaction time in this experiment was set for 1 hour.

Assay buffer

$CaSO_4$	0.2 mM
MES buffer pH 5.5	5.0 mM

Photographs showing confirmation of ferric-chelate reductase activity are shown in Fig. 16 and Fig. 17. Photograph showing confirmation of ferric-chelate reductase activity of the second generation plant is shown in Fig. 18. Reduction of Fe(III) was confirmed as a result of coloring of the transformant in the rhizosphere.